

SECRETION<sup>1</sup> OF INULIN, XYLOSE AND DYES AND ITS  
BEARING ON THE MANNER OF URINE-FORMATION  
BY THE KIDNEY OF THE CRAYFISH

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INTRODUCTION

The following data are the outcome of an attempt to find whether filtration occurs through the nephron of the crayfish. While they do not conclusively exclude filtration, they are sufficiently interesting to warrant presentation.

In contrast to the glomerular kidney of vertebrates, the aglomerular vertebrate kidney cannot eliminate glucose, even during hyperglycaemia and phloridzination (Marshall, 1930), and cannot eliminate xylose (Jolliffe, 1930), or inulin (Richards, Westfall, and Bott, 1934). Furthermore, there is little or no doubt that inulin is not secreted by nor passively resorbed through the vertebrate nephron (see Smith, 1937). Accordingly, it was presumed that the presence or absence of inulin in the urine of the crayfish (a classical freshwater invertebrate), after its injection into the haemocoel, would demonstrate whether filtration occurs.

It is here shown that although inulin and xylose do appear in the urine of the crayfish, they are, at least in part, actually secreted. It is therefore unnecessary to invoke filtration to explain the excretion of these carbohydrates. The ability of all parts of the nephron of this animal to secrete or accumulate one dye or another (see below) and of the coelomosac to secrete calcium (Maluf, 1941a) indicates that this nephron is mainly, if not entirely, a secretory organ.

The subject is *Cambarus clarkii*, which frequents the freshwater swamps of southern Louisiana.

<sup>1</sup> Throughout this paper, *secretion* implies the transport of a substance from a region of lower to one of higher diffusion potential for that substance. *Excretion* refers merely to the outward elimination of undesirable material, regardless of whether the latter is secreted or filtered.

This work was begun in the Department of Zoology, The Johns Hopkins University, while the author was Johnston Research Scholar. Many thanks are due to Prof. S. O. Mast for numerous kindnesses and appreciative criticism.

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## METHODS

*Inulin*

*Analysis.*—The concentration of inulin in the blood and urine was measured, after acid-hydrolysis, by the Shaffer-Hartmann-Somogyi method (see Shaffer and Somogyi, 1933) using Shaffer-Hartmann reagent "50" and Somogyi's (1931) procedure for deproteinization of the serum. The technique was adapted to the small quantities used in this work as follows. Blood was taken by amputating a leg at the femur and allowing about 0.15 cc. to run into a small test-tube ( $9 \times 75$  mm.). Bleeding was instantly and permanently stopped by compressing the stump with the hot tips of a blunt forceps. The test-tube was stoppered and heated at  $80^\circ$  C. for about a minute or until the blood became opaque and was then cooled rapidly. The resulting solid was broken up with a fine glass-rod and the tube centrifuged for a few minutes. A sample of the supernatant liquid, usually about 75 cu.mm. was drawn into a fine calibrated pipette of the constricted type (Fig. 1, *A*). It was deproteinized by adding an equal volume of 7 per cent  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and another equal volume of 10 per cent  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ . Distilled water was added according to the required dilution (5 to 16 times), the same pipette being used in adding the water and reagents as that in taking the sample. After stoppering, shaking, and permitting to stand for at least 20 minutes, the tubes were centrifuged and 80 cu.mm. aliquots drawn for analysis. About 150 cu.mm. distilled water were added to increase the volume and then about 70 cu.mm.  $\text{N H}_2\text{SO}_4$  for hydrolysis. The tubes were capped with glass-bulbs and heated in rapidly boiling water for 15 minutes. After cooling, a small drop of phenolphthalein was added. The solutions were neutralized with  $\text{N KOH}$ . If the color became too intense it was brought to pink with 0.1  $\text{N H}_2\text{SO}_4$ ; 0.161 cu.mm. of the Shaffer-Hartmann reagent was added and then a few drops of distilled water to augment the volume to about 1 cc. The test-tubes were shaken, capped with glass-bulbs, and heated without agitation in rapidly boiling water for 15 minutes. After cooling, the cap was removed only just before the contents of that tube were to be titrated and about 250 cu.mm.  $\text{N H}_2\text{SO}_4$  introduced. The solid was completely dissolved with a glass-rod without undue agitation and the contents titrated with 0.01  $\text{N Na}_2\text{S}_2\text{O}_3$  until the color, due to the free  $\text{I}_2$ , became a very light yellow. About 35 cu.mm. of a 1 per cent aqueous solution of starch were added and the titration continued to the end-point. Titration was from a Linderström-Lang-Keys microburette of 250 cu.mm. capacity, divided into cubic millimeters, and of uniform bore as shown by measurements of the length of a drop of mercury at all levels. The concentration of

inulin was ascertained by interpolation in a graph established from aqueous solutions containing a known quantity of the inulin (Pfanstiehl inulin, c.p.). Blanks and standards were run frequently. To obtain values with respect to the plasma, 5 per cent was deducted from the ascertained value, this being the approximate quantity of total solids in the whole blood of the crayfish and presumably close to the quantity which fell out by heating the blood at 80° C. The accuracy was within 5 per cent of the amount present.

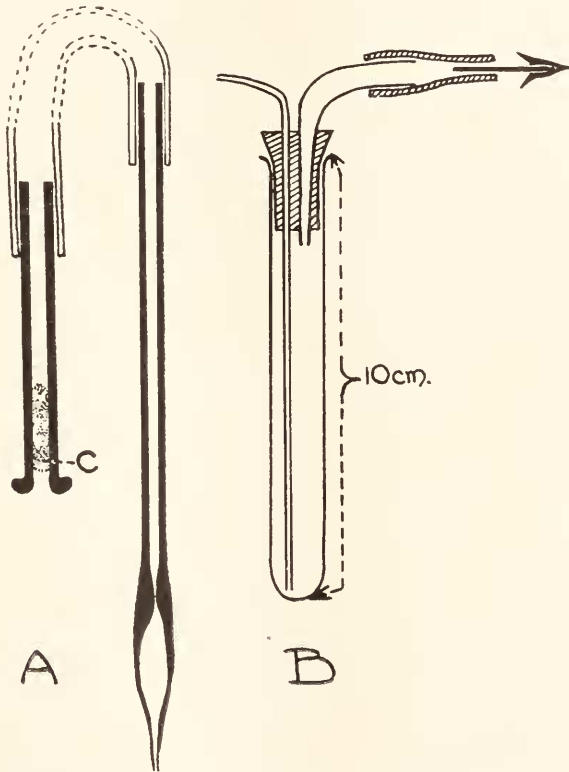


FIG. 1. *A.* Volumetric micro-pipette of the constricted type with mouth-piece and rubber tubing. *c.* cotton-plug. *B.* Apparatus for the collection of urine and emptying of bladders. Arrow indicates direction of suction.

The urine was treated in the same manner as the blood with the exceptions that heating and deproteinization were unnecessary, the urine being protein-free, and that, in the first 26 experiments, a correction for evaporation had to be applied (see below).

The preparation of inulin contained some impurity which, without being hydrolyzed, reduced Benedict's qualitative and which could not be

removed by yeast. The Pfanstiehl Company advised us that they had not been able to eliminate the impurity by repeated crystallization. The impurity was, however, negligible because within less than an hour after the injection of inulin, in the quantities used in this work (see Table II), the blood (whole or protein-free) was non-reducing unless subjected to acid-hydrolysis. Evidently the tissues removed the reducing substance rapidly. At no time could the urine reduce Benedict's reagent without preliminary acid-hydrolysis. Yeast-adsorption was therefore unnecessary in the analysis of inulin. Possibly because the animals were starved for a few days, the blood (whole or protein-free) of unsubjected animals was non-reducing even with preliminary acid-hydrolysis; the urine of these animals was invariably non-reducing. Crayfish can endure, without appreciable injury, starvation for four months at least (Brunow, 1911).

*Inulin-clearance.*—The renal clearance of a substance has a definite physiological meaning, being the virtual volume of blood cleared of that substance per unit time by the kidneys. It is expressed by  $C = UV/P$ , in which  $C$  is the clearance,  $P$  the concentration of the substance in the plasma, and  $V$  the rate of urinary flow. It is necessary to know the average concentration of that substance in the blood throughout the time that the urine to be analyzed is being formed.

Before an experiment, the crayfish was kept overnight fully submerged in running aerated freshwater. In measuring the inulin-clearance, the integument and branchial chambers were drained of moisture, the animal was weighed, and a fraction of a cc. of crayfish-saline<sup>3</sup> containing a given quantity of inulin injected slowly through the proximal abdominal venter and the wound cauterized. The amount of inulin injected was adjusted mainly by altering the concentration of the dissolved inulin in the saline because it was not desired to augment the blood-pressure by injecting a relatively large quantity of liquid (Table II). By thorough bleeding, the total quantity of blood in an average-sized *Cambarus clarkii* was found to be 6.6 per cent of the wet weight, which corresponds closely to the 6.7 of Herrmann (1931), who used the same method with *Potamobius astacus*.

After about 45 minutes the animal, including its branchial chambers, was drained of moisture and the anterior margins of the latter plugged with cotton-wool. The bladders were emptied by suction (about 12 mm. Hg) applied at the nephropores through the arrangement in Fig. 1, B.

<sup>3</sup>The saline was based on the most acceptable data on the concentration of inorganic electrolytes in the blood of the crayfish (see Maluf, 1940, for references) and was as follows (g./l.): NaCl, 7.81; CaCl<sub>2</sub>, 1.31; MgCl<sub>2</sub>, 0.82; KCl, 0.70; buffered at pH 7.6 by 0.5 cc. M/5 Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>. This assumes a  $\Delta$  of about 0.66° C. (see Lienemann, 1938, and Schlatter, 1941).



When no urine could be obtained, firm bilateral digital pressure was applied to the integument lateral to the bladders (Maluf, 1941*a*) and suction again used until no further urine issued. The first sample of blood was taken immediately after and at what was considered zero time. The concentrations of inulin in blood from the pericardial sinus and from a leg were practically identical at that time, thus showing uniform distribution of the foreign material. The nephropores were cauterized to ensure a dry surface. "Ames Temporary [dental] Cement:

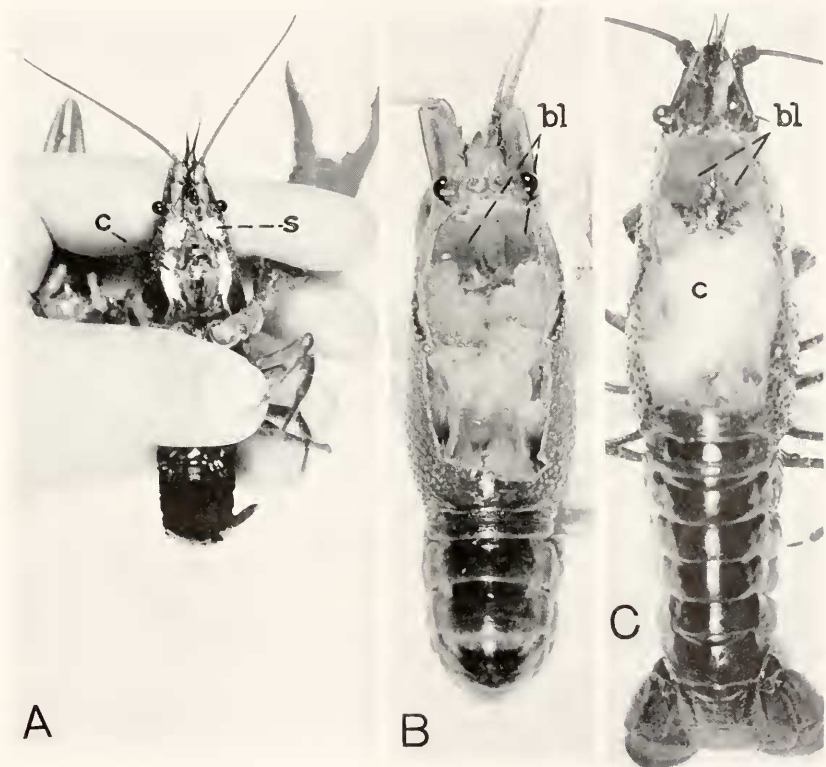


FIG. 2. *A*. Manner of handling the crayfish while the bladders are emptied and the nephropores are being sealed. *c*, cotton-plug in anterior margin of right branchial chamber; *s*, seal on the basal segment of the left antenna. *B*. Dorsal aspect of a crayfish with dorsal part of carapace and crop-gizzard removed, showing both bladders, *bl*, distended. *C*. Same as *B* but with viscera moved posteriorly and with wad of cotton, *c*, between viscera and bladders, *bl*.

hydraulic, non-irritant" was applied to the excretory eminence and basal segment of the antenna in two layers, under a magnification of  $10.5\times$ , by means of a forceps. The animal was handled as shown in Fig. 2, *A*. The cement should not extend beyond the basal antennal segment because movement of the antenna would be likely to crack the dried seal. Hard-

ening, which is due to the formation of zinc phosphate from zinc oxide and phosphoric acid, is rapid. Other cements were tried but were incomparably inferior. Ten minutes after completing the application the animal was fully immersed in freshwater and kept undisturbed.

Three or four blood-samples were taken through the experiment, which lasted 8 to 15 hours. Immediately after the last sample, the ventral nerve-cord was transected at the proximal level of the abdomen so as to prevent abrupt abdominal flexion, the chelipeds were amputated basally, and the dorsal surface of the carapace and the crop-gizzard, which is wedged over and between the bladders, were carefully removed. The distended bladders presented themselves conspicuously (Figs. 2, *B* and *C, bl*). The urine contained in the translucent bladders was crystal-clear and the kidneys could be seen beneath (Fig. 2, *C*). The viscera were then pushed back and a wad of absorbent cotton (Fig. 2, *C, c*) was applied over them to keep any fluid from flowing near the bladders. The urine was rapidly and completely collected by applying suction (about 12 cm. Hg) through the orifice of the arrangement shown in Fig. 1, *B* to the surface of each bladder. The animal was tipped on the side of collection with the head downward while this was done. The rate of urinary flow was thus accurately measured. The rapid collection obviated a correction for loss by evaporation. In the very few instances in which both bladders were not equally distended, the kidney corresponding to the lower rate of urinary flow was diminutive in size.

In the first 26 experiments on inulin-clearance the nephropores were not sealed because it was assumed that undisturbed animals with emptied bladders would not urinate appreciably during the interval. Urine, in these instances, was collected by suction from the nephropores and a correction applied for the fraction of water lost by evaporation. This is quite appreciable, and because previous investigators have not taken it into account the writer has no doubt that their values for the concentration of solutes in the urine of the crayfish are higher than the actual. The necessary corrections were obtained by aspirating, with the same pressure, a known quantity of distilled water, from the tip of a fine pipette of the constricted type (Fig. 1, *A*), into a test-tube of the same dimensions as that used for the collection of urine (Fig. 1, *B*). The resultant quantity of water, after light centrifugation of the test-tube for a few seconds, was measured by drawing it into a calibrated glass-tube. The loss in collecting 0.148 cc. in two minutes was 16.4 per cent and that in collecting 0.0739 cc. in five minutes was 35 per cent. The first correction was the one generally applied, as it was the writer's policy to collect the maximal quantity of urine in the minimum time with the above pressure. Generally an amount of 0.15 to 0.2 cc. could be

readily collected within two minutes. A successful and rapid collection depends to a great extent upon the aspirating tip. This should not have sharp edges but should be blunt and regular; its diameter should not be so large as to cover the entire excretory eminence. There is no doubt that the water lost was due entirely to evaporation. Scrupulous care was taken to prevent water-contamination of the urine, by draining the animal thoroughly, sucking water from the branchial chambers and rostral region, and plugging the anterior margins of the chambers with cotton-wool. The urine was not contaminated with blood, as was shown by negative biuret-, heat-, and  $H_2SO_4$ -tests, by an uninjured operculum, and by the fact that the concentration of inulin and dyes in the urine was considerably greater than in the blood (see below).

The rate of urinary flow was not measured in the first 26 experiments. It was assumed to be constant from one individual to another per unit weight, being determined, in a fully submerged animal, by the rate of diffusion of water into the body (Herrmann, 1931). Otherwise the rate of flow was measured by the above technique which necessitates sacrificing the animal at the end of the experiment. In ten experiments with inulin (Table I), the rate averaged 5.0 cc. per 100 grams per 24 hours. This is quite close to the average ( $=5.2$ ) of Lienemann (1938), who collected the urine by aspiration from nephropores which had been sealed, and was taken as the rate of flow for the animals in the first 26 experiments. The rate of urinary flow in the crayfish is low as compared with the frog (Forster, 1940) and freshwater turtle (Friedrich, Holman, and Forster, 1940), and even relative to that in birds and a terrestrial reptile (Marshall, 1932). This emphasizes the low permeability of the gills of the crayfish to water.

The inulin-clearances and  $U/P$ 's obtained through the use of direct measurements of urinary flow with sealed nephropores (Figs. 6 and 7, *inulin*; solid circles) and from an average rate of flow with unsealed nephropores (Figs. 6 and 7, *inulin*; open circles) are quite comparable.

The average concentration of inulin in the blood through the experimental period was secured by averaging the interpolated values at the mid-period of each hour (see curves representing concentration-time, Fig. 3). Three or four blood-samples were sufficient to establish the shape of the curves. Furthermore, it was undesirable to take more blood than necessary.

#### *Xylose*

The analysis of xylose was identical with that of inulin except that acid-hydrolysis was omitted.

The nephropores were sealed and the rate of urinary flow measured directly (see above and Table I).

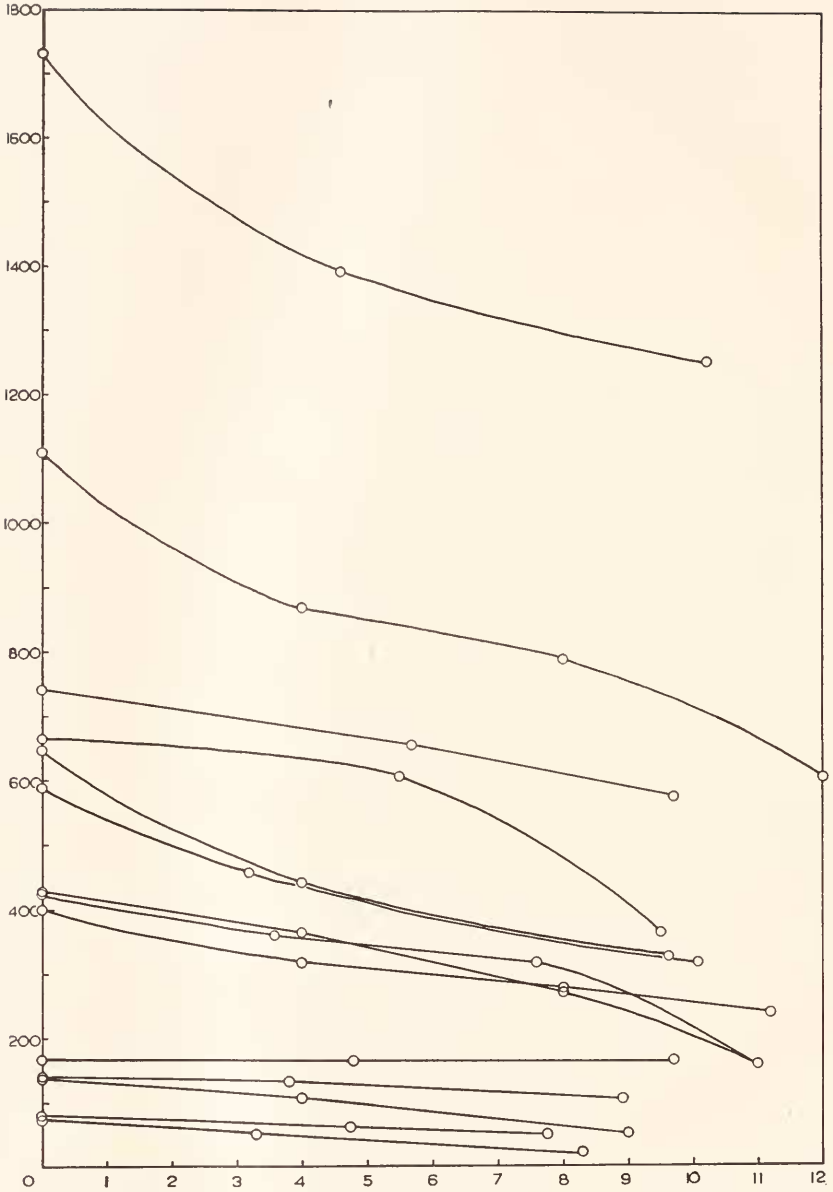


FIG. 3. The concentration of inulin in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during measurements of inulin-clearance.

Three blood-samples were sufficient (Fig. 4). The average concentration of xylose in the blood through the experimental period was calculated in the same way as for inulin.

### *Creatinine*

Deproteinization of the serum was unnecessary because of the large dilution (about  $26\times$ ). To 80 cu.mm. of serum or urine were added 2 cc. of distilled water. The tubes were capped and shaken and 1 cc. of the alkaline picrate was added to each. A Dubosque-type colorimeter with

TABLE I  
*Urinary flow in cc. per 100 grams per 24 hours.*

No.	Male	Female	Material injected
1		3.4	0.3 cc. 10% inulin in crayfish-saline.
2		4.5	0.4 cc. 10% " " " "
3		3.3	1 cc. 20% " " " "
4		4.4	0.4 cc. 5% " " " "
5		6.1	0.4 cc. 5% " " " "
6	5.4		0.6 cc. 10% " " " "
7	6.4		0.6 cc. 10% " " " "
8		8.8	0.2 cc. 5% " " " "
9		4.4	0.2 cc. 5% " " " "
10	3.7		0.2 cc. 5% " " " "
11		5.4	1 cc. 30% xylose in $\frac{2}{3}$ crayfish-saline.
12	7.2		1 cc. 30% " " " "
13	7.9		0.3 cc. 10% xylose in dist. water.
14		8.85	0.2 cc. 10% " " " "
15	7.1		0.5 cc. 10% " " " "
16	4.6		0.5 cc. 10% xylose in crayfish-saline.
17		2.9	0.5 cc. 5% creatinine in crayfish-saline.
18	4.6		0.5 cc. 5% " " " "
19		5.9	0.2 cc. 5% " " " "
20		5.7	0.2 cc. 5% " " " "
21	7.6		0.5 cc. 10% creatinine in dist. water.
22		8.9	0.5 cc. 10% " " " "
23	2.9		0.5 cc. 15% creatinine (somewhat toxic) in dist. water.
24		3.1	0.5 cc. 15% " " " " " "

1-cc. cups was used. The light was passed through a green filter. Standards were made each time as expected. There was never as much as a 50 per cent difference between the samples and the standards. The blood of unsubjected animals did not give a positive Jaffé reaction.

The blood-curves were almost straight lines (Fig. 5). The average concentration of creatinine in the blood through the experimental period was calculated in the same way as for inulin.

The nephropores were sealed and the rate of urinary flow measured directly (see above and Table I).

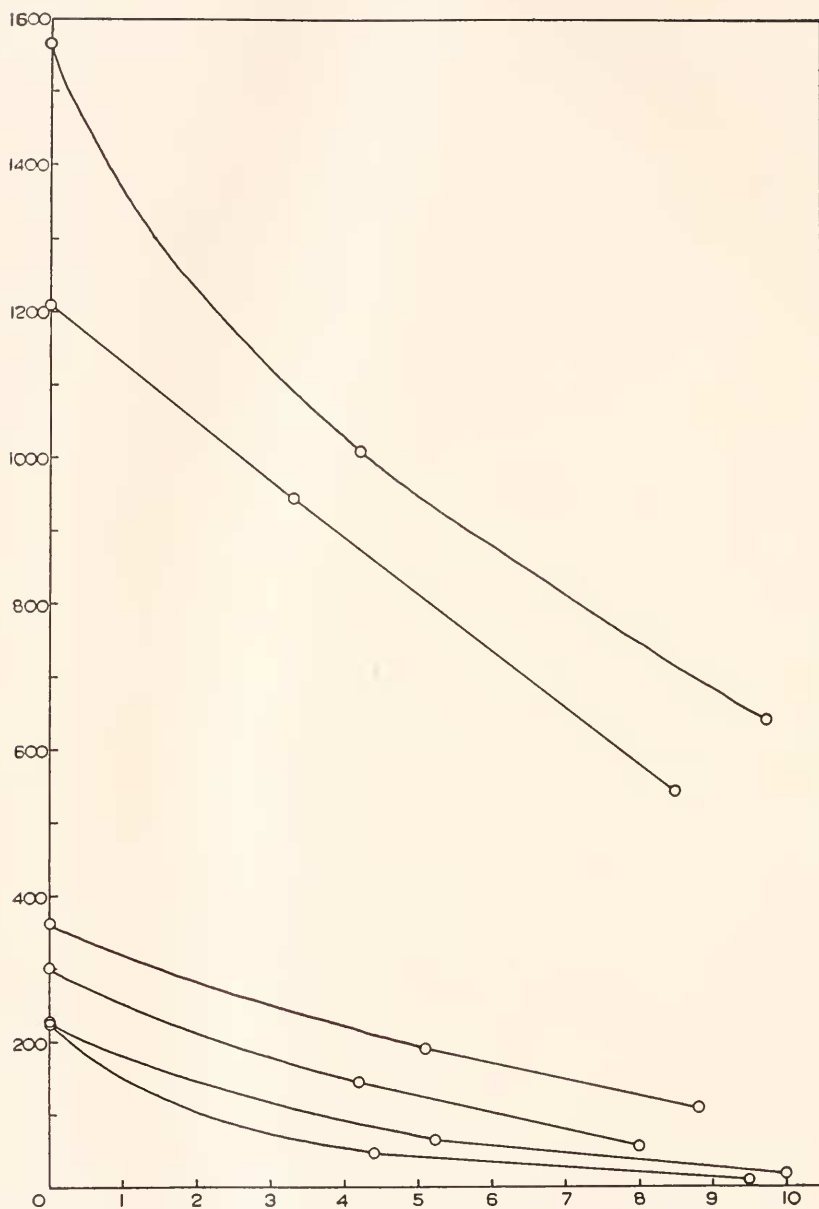


FIG. 4. The concentration of xylose in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during the measurements of xylose-clearance.



## RESULTS

*Excretion of Inulin*

In all the preliminary experiments, the injection of 0.2 cc. 5 per cent inulin in crayfish-saline resulted in a renal output of inulin. Thus, the urine, which was collected by suction from the nephropores, gave a positive result with Benedict's qualitative only after acid-hydrolysis. Before the introduction of inulin the urine was non-reducing even with acid-hydrolysis.

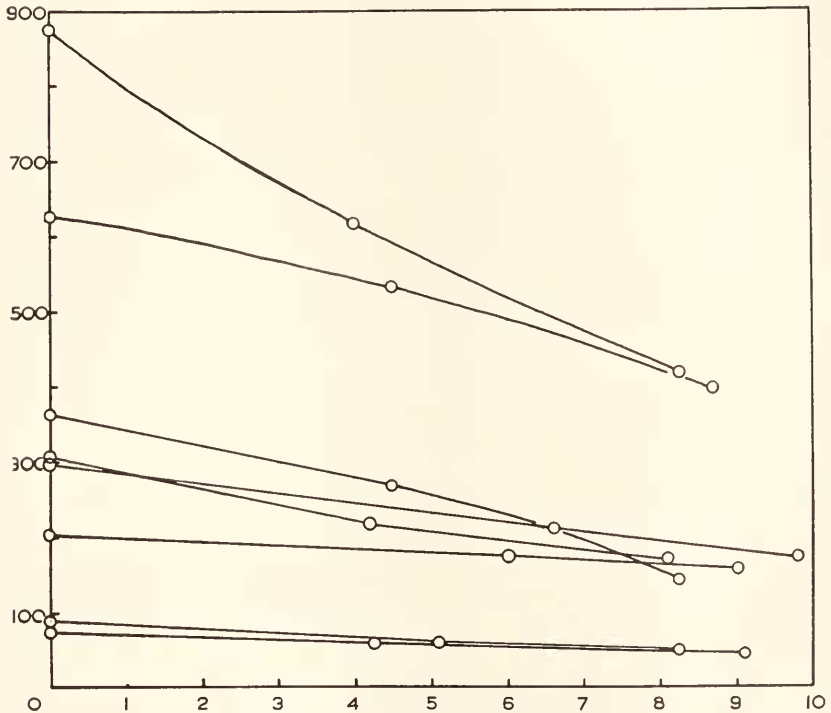


FIG. 5. The concentration of creatinine in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during the measurements of creatinine-clearance.

Similar results followed the injection of xylose or glucose. To guard against possible glucose-contamination in the sample of xylose, the urine was shaken for a few minutes with an equal quantity of a 20 per cent suspension of washed yeast in distilled water and centrifuged. Blanks, with only the yeast-centrifugate, were non-reducing. The glucose was given in high concentration (0.6 cc. 70 per cent per 40 grams) because the tissues tended to remove it from the blood.

Because glucose, xylose, and inulin are excreted by the kidney of the crayfish and not by the vertebrate aglomerular kidney, it at first seemed that filtration occurs in the former. This might imply that the hypotonicity of crayfish-urine is produced as in the Amphibia, namely, by the formation of a protein-free filtrate at the proximal end of the nephron and by subsequent resorption of relatively more salts than water by the tubule. On the other hand, other important data (see Discussion) contra-indicate filtration.

Because inulin is neither secreted by nor passively resorbed through the vertebrate nephron, the inulin-clearance in this phylum is an unvarying function of the concentration of inulin in the plasma. This is true

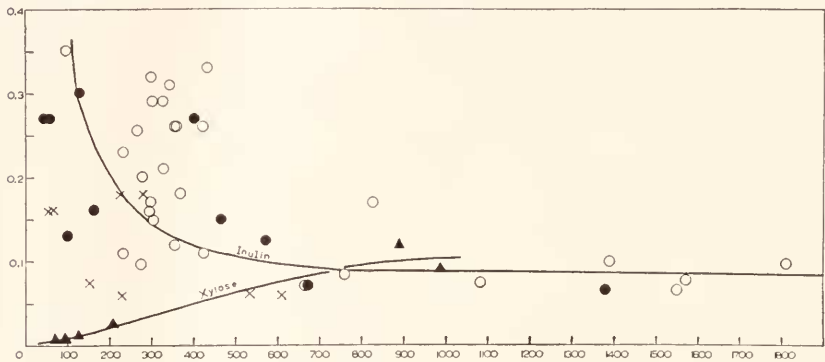


FIG. 6. The renal clearance of inulin, xylose, and creatinine in cc. per hour (ordinate) as a function of the concentration of these compounds in the plasma in mg. per cent (abscissa). *Solid circles*, inulin-clearances with direct measurement of urinary flow and nephropores sealed; *open circles*, inulin-clearances without direct measurement of urinary flow and nephropores not sealed; *triangles*, xylose-clearances with direct measurement of urinary flow and nephropores sealed; *crosses*, creatinine-clearances with direct measurement of urinary flow and nephropores sealed. Each point stands for a single separate animal.

even at the low plasma-concentrations (Miller, Alving, and Rubin, 1940). To find whether secretion can account for the marked occurrence of inulin in the urine of the crayfish, a study was made of the inulin-clearance at various levels of inulin in the plasma. Variation of the renal clearance with the plasma-concentration would demonstrate secretion. Parenthetically, even if the renal clearance of a substance does not vary with its plasma-concentration, secretion is not theoretically excluded (Shannon, 1938, 1939).

The actual inulin-clearance: plasma-inulin relationship (Fig. 6, *inulin*) demonstrates an outward secretion of inulin. The  $U/P$ : plasma-inulin curve (Fig. 7, *inulin*) is similar and the  $U/P$ 's were above unity. Because the renal clearance is a product of the  $U/P$  and rate of urinary

flow, the approximate identity in the curves of Figs. 6 and 7 is equivalent to stating that the rate of urinary flow tends to be constant among different individuals.

It should be pointed out that the wet weight of both kidneys is normally a direct rectilinear function of the wet weight of the crayfish, at least in animals weighing between 10 and 50 grams. The relationship is expressed by  $y = 0.0026x$ , in which  $y$  is the mass of both kidneys and  $x$  the mass of the entire animal. Because the inulin-secreting mass of the kidney is probably a direct function of the total mass of the kidney, all animals should be approximately the same weight in an ideal set of experiments. In this investigation, because the lower plasma-concentrations were by no means confined to the larger animals (Table II), size, within the experimental range, cannot have been a determining factor in the inulin-clearance:plasma-inulin relationship. This is further brought out by the fact that the variation in the inulin-clearance with the concentration of inulin in the plasma, in crayfish which range between average and large size, is determined practically entirely by the  $U/P$  (Figs. 6 and 7, *inulin*) and not by the volume of urine excreted, which is greater in the larger animals although fairly constant per unit weight. In other words, the hourly differences in the absolute rate of urinary flow among individuals of somewhat different size are relatively small and inconsistent as compared with the variation of the  $U/P$  with the concentration of inulin in the plasma. Assuming a constant concentration of inulin in the plasma, the inulin-clearance ( $= UV/P$ ) would doubtless vary with the mass of the kidney, but the  $U/P$  probably would not because the inulin-secreting mass of the kidney may bear a constant value with respect to the water-secreting mass. This implies that while the large kidney would secrete more inulin than the small one, it would also secrete proportionally more water.

The shape of the  $U/P$ :plasma-inulin curve indicates that the renal cells asymptotically become functionally saturated with inulin as the plasma-level of this compound rises. If filtration does not occur one would expect that, at *extremely* low concentrations of inulin in the plasma, the  $U/P$ 's would be less than unity because there would be very little inulin available to the renal cells within a given interval of time. Apparently because of the relatively high avidity of the renal cells for inulin, it was not practicable to measure inulin-clearances at extremely low plasma-levels; sufficient urine was not formed before the blood was freed from inulin. With xylose,  $U/P$ 's below unity occur even at moderate plasma-levels. It is possible that, at moderate concentrations, the kidneys secrete relatively more inulin than water and that the reverse is true for xylose.

Attempts were made to locate the site of inulin-secretion in the nephron by the colorimetric method of Alving, Rubin, and Miller (1939). About 0.8 cc. 10 per cent inulin in crayfish-saline were injected into

TABLE II  
*Excretion of Inulin*

No.	Wgt. in g. and sex	Inulin		Urinary flow	Amt. of crayfish saline and conc. of inulin injected	Duration of experiment
		Plasma	Urine			
		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>cc./hr.</i>		<i>hrs.</i>
1	42.7 ♀	433	1,590	0.088	0.6 cc. 10%	12.5
2	30.0 ♀	345	1,716	0.062	0.25 cc. 10%	11.5
3	27.8 ♀	355	1,600	0.058	0.27 cc. 10%	13.75
4	42.5 ♀	1,810	1,970	0.088	1 cc. 20%	14.25
5	34.2 ♂	1,390	1,974	0.072	1 cc. 20%	14.75
6	31.0 ♂	99	540	0.064	0.2 cc. 5%	13.5
7	25.7 ♂	234	468	0.054	0.2 cc. 5%	13.7
8	33.0 ♀	1,086	1,246	0.068	0.75 cc. 10%	12.5
9	42.7 ♂	235	578	0.090	0.4 cc. 5%	13
10	44.8 ♂	296	494	0.094	0.4 cc. 5%	12.6
11	33.4 ♂	758	883	0.070	0.5 cc. 10%	11.3
12	23.1 ♀	276	550	0.048	0.17 cc. 10%	11.5
13	51.1 ♂	304	825	0.106	0.38 cc. 10%	11
14	37.3 ♂	280	742	0.076	0.28 cc. 10%	13
15	51.7 ♂	356	841	0.108	0.45 cc. 10%	12
16	26.0 ♀	359	825	0.054	0.26 cc. 10%	12.3
17	28.0 ♂	299	858	0.058	0.28 cc. 10%	12.4
18	25.0 ♀	1,568	2,350	0.052	0.5 cc. 20%	11.6
19	23.0 ♀	1,549	2,130	0.048	0.5 cc. 20%	12
20	46.5 ♂	307	494	0.096	0.65 cc. 10%	14
21	48.5 ♂	425	462	0.10	0.68 cc. 10%	12.5
22	27.6 ♀	830	1,155	0.12	0.7 cc. 10%	12
23	21.7 ♀	330	718	0.094	0.2 cc. 10%	11
24	25.0 ♂	330	882	0.108	0.3 cc. 10%	10.6
25	20.4 ♀	300	1,090	0.088	0.4 cc. 10%	11.2
26	21.4 ♂	370	1,156	0.092	0.4 cc. 10%	11.2
27	28.0 ♀	670	1,180	0.040	0.3 cc. 10%	9.7
28	33.5 ♀	574	1,160	0.06	0.4 cc. 10%	9.5
29	30.3 ♀	1,380	2,220	0.042	1 cc. 20%	10.2
30	26.5 ♀	166	530	0.049	0.4 cc. 5%	9.8
31	29.5 ♂	131	520	0.075	0.4 cc. 5%	8.9
32	47.3 ♂	467	720	0.106	0.6 cc. 10%	9.7
33	32.0 ♂	424	1,250	0.089	0.6 cc. 10%	10.6
34	23.8 ♀	60	192	0.088	0.2 cc. 5%	7.75
35	29.5 ♀	102	240	0.054	0.2 cc. 5%	9
36	42.0 ♂	45	180	0.065	0.2 cc. 5%	8.3

medium-sized animals. After about three hours the kidneys were removed, rinsed in saline, and the coelomosac, tubule, and labyrinth teased apart. Approximately equal amounts of coelomosac, labyrinth, and tubule were put into separate small test-tubes. To each was added 1

cc. of the freshly prepared diphenylamine reagent. The tubes were capped and put into a boiling water bath for six minutes. The color which developed at the end of this time was evidently maximal. Unaided visual examination of the intensity of color did not indicate any differences in the amount of inulin present in the tubes. The intensity was determined solely by the mass of tissue used.

Regardless of whether a substance is removed from the blood by extrarenal tissues, the renal clearance of the substance will be a function of the concentration of that substance in the blood. It was nevertheless of interest to find if inulin can be hydrolyzed by the tissues of the cray-

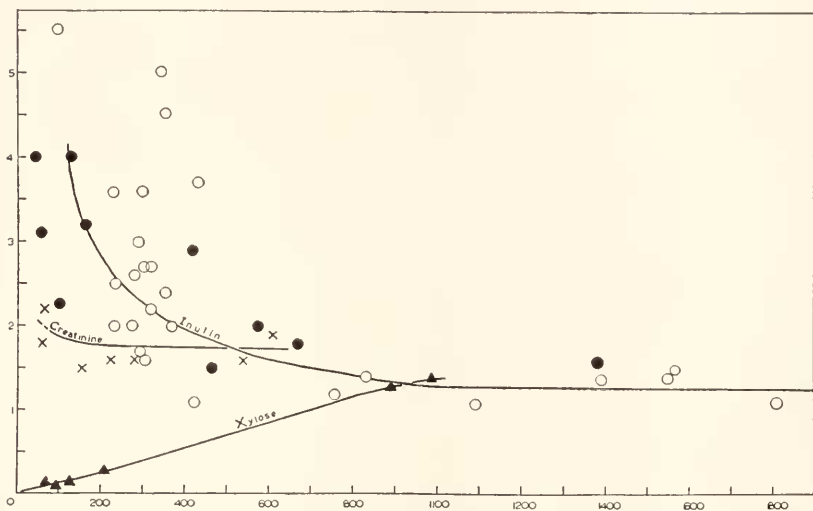


FIG. 7. The  $U/P$  ratio of inulin, xylose, and creatinine (ordinate) as a function of the concentration of these compounds in the plasma (abscissa). Notations identical with those in Fig. 6. Each point stands for a single separate animal; the same as those in Fig. 6.

fish. The kidneys and samples of the hepatopancreas, somatic muscles, and blood were frozen in solid carbon dioxide, thoroughly macerated, and extracted in a known quantity of saline. To aliquots of the centrifugates were added a solution of inulin and a small drop of xylol. The mixtures were analyzed for inulin immediately and after 13 hours at room temperature. The controls contained only a solution of inulin and the preservative. There was no change in the concentration of reducing carbohydrate, with or without acid-hydrolysis, in any tube. This indicates that, under the conditions of the experiments at least, inulin is not hydrolyzed by the tissues of the crayfish. Similar experiments showed a destruction of d-xylose in the following descending

order: hepatopancreas, kidneys, somatic muscles, blood. This may explain how the concentration of xylose in the blood falls more rapidly than that of inulin (Figs. 3 and 4) even though the renal xylose-clearances (see below) are lower than the inulin-clearances. Xylose may also diffuse out through the gills.

#### *Excretion of d-Xylose*

The xylose-clearance varies directly with the concentration of xylose in the plasma (Fig. 6, *xylose*) and at moderately low plasma-levels the *U/P*'s are well below unity (Fig. 7, *xylose*). Assuming the occurrence of filtration and resorption, this relationship may be explained by an incapacity of the nephron to resorb xylose beyond a maximal rate; as a

TABLE III  
*Excretion of d-Xylose*

No.	Wgt. in g. and sex	Xylose		Urinary flow cc./hr.	Remarks
		Plasma <i>mg. per cent</i>	Urine <i>mg. per cent</i>		
1	29.6	988	1,370	0.067	1 cc. 30% xylose in $\frac{2}{3}$ crayfish-saline; 9.75 hr. duration.
2	38.1	890	1,120	0.115	1 cc. 30% xylose in $\frac{2}{3}$ crayfish-saline; 8.5 hr. duration.
3	31.5	89	10	0.10	0.3 cc. 10% xylose in dist. water; 10 hr. duration.
4	22.5	64	10	0.083	0.2 cc. 10% xylose in dist. water; 9.5 hr. duration.
5	33.6	210	61	0.10	0.5 cc. 10% xylose in dist. water; 8.8 hr. duration.
6	34.5	127	19	0.066	0.5 cc. 10% xylose in crayfish-saline; 8 hr. duration.

consequence, an increasing amount would "spill over" as the plasma-level is raised. Because at low plasma-concentrations the *U/P* is below unity (Fig. 7, *xylose*) the resorption would presumably be active, i.e. xylose would be inwardly secreted. There is a resemblance to the handling of glucose and other threshold-substances by the mammalian kidney.

On the other hand, the process can be readily explained, without resort to filtration, by assuming that both xylose and water are outwardly secreted and that, at low plasma-levels of xylose, the rate of secretion of water is relatively large compared with the secretion of xylose. At moderately high plasma-levels the xylose-clearance is nearly identical with the inulin-clearance (Fig. 6). In the experiments which necessitated the introduction of sufficient xylose to raise the average



plasma-concentration to about 1000 mg. per cent (see Table III), the animals became torpid soon after the injection but recovered completely within several minutes. It was therefore not considered within the scope of a physiological experiment to measure xylose-clearances at still higher plasma-levels. The injurious effects are probably osmotic. Inulin was not toxic even at the high concentrations.

For the same reason as with inulin, the xylose-clearance: plasma-xylose curve is practically identical with the *U/P*: plasma-xylose curve (Figs. 6 and 7, *xylose*).

TABLE IV  
*Excretion of Creatinine*

No.	Wgt. in g. and sex	Creatinine		Urinary flow cc./hr.	Remarks
		Plasma	Urine		
		<i>mg. per cent</i>	<i>mg. per cent</i>		
1	25.5 ♀	230	460	0.031	0.5 cc. 5% creatinine in crayfish-saline; 9.8 hr. duration.
2	26.5 ♂	155	240	0.051	0.5 cc. 5% creatinine in crayfish-saline; 9 hr. duration.
3	30.0 ♀	65	140	0.074	0.2 cc. 5% creatinine in crayfish-saline; 9.1 hr. duration.
4	39.0 ♀	60	105	0.093	0.2 cc. 5% creatinine in crayfish-saline; 8.25 hr. duration.
5	34.7 ♂	280	450	0.112	0.5 cc. 10% creatinine in dist. water; 8.25 hr. duration.
6	32.0 ♀	225	350	0.118	0.5 cc. 10% creatinine in dist. water; 8.1 hr. duration.
7	33.8 ♂	537	850	0.040	0.5 cc. 15% creatinine in dist. water; 8.7 hr. duration. (Somewhat toxic.)
8	25.5 ♀	610	1,150	0.033	0.5 cc. 15% creatinine in dist. water; 8.25 hr. duration. (Somewhat toxic.)

*Excretion of Creatinine*

Because the inulin- and creatinine-clearances are identical in certain vertebrates at all plasma-levels, it was desirable to compare the same clearances in the crayfish. The results were not elucidating and are presented here merely for record because it is believed that they are accurate (Figs. 6 and 7, *creatinine*; Table IV). Plasma-concentrations higher than 900 mg. per cent were definitely injurious if not fatal. The maximal ones on record are just within the threshold of toxicity, judging from the activity of the animal.

*Excretion of Dyes*

The initial objective of the experiments under this heading was to find if the nephron of the crayfish is capable of eliminating dyes which

the vertebrate glomerular kidney is incapable of excreting. It was also desirable to study the capacities of the different parts of the nephron to secrete or accumulate various kinds of dyes.

The dyes were dissolved in crayfish-saline immediately before use. A description of the chemical composition of most of the dyes can be found in Conn's (1925) monograph.

*Cyanol* (DuPont).<sup>4</sup>—This is an aniline dye giving an intense blue in solution even when very dilute. Cyanol is not eliminated by the glomerular vertebrate kidney if given in doses of the order of several mg. per kg. (Höber, 1930) but is slightly excreted when in quantities of 125–300 mg./kg. (Marshall and Grafflin, 1932).

Immediately after emptying the bladders, a fraction of a cc., containing a dose of about 1.7 mg./kg., was injected through the proximal abdominal venter. This colored the blood a vivid blue. Urine was collected after five hours and had to be diluted about tenfold to bring the intensity of color down to that of blood taken only one hour after the injection. Within five hours the blood lost all trace of blue. The experiment was repeated with similar results. As stated above, the concentration of foreign material, one hour after injection, is about equal in blood taken from the legs as in that from the pericardial sinus.

Other subjects were opened one to two hours after the injection. The viscera were rinsed with saline. Cyanol was not found in any organ other than the labyrinthic epithelium. The intensity of blue in the labyrinth not only greatly exceeded that of blood at the time but even that of blood taken only twenty minutes after the injection. The dye did not stain the bladder nor diffuse out from the contained urine even at a time, five or six hours after the injection, when it was absent from the blood.

The accumulation of cyanol in the labyrinth cannot be considered due to a resorption of water by the labyrinth, from a filtrate conceivably formed at the coelomosac, because: (1) The dye is greatly concentrated in the labyrinthic cells and yet not appreciably apparent in the more distal parts of the nephron; (2) the labyrinthic cells, even in "living" hanging-drop preparations, indicate a marked outwardly secretory activity as shown by the frequent presence of globules apparently being pinched off toward the lumen: the labyrinth therefore can scarcely be considered as a water-resorbing organ from a cytological standpoint; (3) the data indicate that the coelomosac is a secretory organelle (Maluf, 1941a, and below).

*Ferrocyanide*.—Iron salts, such as ferric ammonium citrate and sodium ferrocyanide, are not excreted by the glomerular vertebrate ne-

<sup>4</sup> Kindly supplied me by Professor E. K. Marshall, Jr.

phron (Marshall and Grafflin, 1932) but are filtered through the glomerular nephron of vertebrates (see Smith, 1937).

Both bladders were emptied and 0.5 to 1.2 cc. of 2.4 per cent sodium ferrocyanide injected into animals weighing from 27 to 48 grams. The Prussian blue color was developed by adding a known quantity of Folin's (1929) ferric sulfate reagent to the  $\text{Na}_2\text{WO}_4\text{-H}_2\text{SO}_4$  protein-free blood-centrifugate. At the end of five hours a scarcely appreciable quantity of urine could be collected, which gave a Prussian blue test. The ferric sulfate reagent produced an intense blue throughout the teased nephron; the color was more intense than that of the blood taken only 0.5 hour after the injection and seen through the same or greater depth. This experiment indicates that the kidney is capable of accumulating ferrocyanide but that the cells apparently become too poisoned to secrete urine. The hepatopancreas, muscles, and alimentary tract, rinsed free from blood and teased apart, gave no reaction.

*Phenol Red.*—Phenol red is secreted by the aglomerular teleost kidney (Marshall and Grafflin, 1932). The bladders of the crayfish were emptied immediately before the injection of the dye. The dose was 1 cc. of 34 mg. per cent phenol red into animals weighing about 30 grams. To develop the maximal intensity of color, both urine and blood were either exposed to  $\text{NH}_3$  or received a known quantity of  $\text{NH}_4\text{OH}$ . The urine, collected five hours after the injection, had to be diluted over ten-fold to reduce its intensity to that of blood taken 20 minutes after. On examining the kidneys in situ five hours after the injection, only the posterior part of the labyrinth had a reddish tinge. On adding a drop of 0.1 N NaOH to the nephron in crayfish-saline, the whole labyrinth became an intense red which was even deeper than that of blood taken as early as 0.5 hour after the injection. The labyrinth is thus capable of secreting phenol red and the pH of its cells is evidently about 7.0. Other tissues, including the coelomosac and nephric tubule, after being briefly rinsed from blood, showed no trace of phenol red.

The urine, as it issued from the nephropore, was a clear orange-red, not the purple-red of maximal intensity, and therefore has a pH of about 7.5.

*Neutral Red.*—In the three animals studied (dose: 1.2–1.8 cc. 80 mg. per cent per 30 grams) there was no indication of a concentration of this dye in the urine. The dye penetrated the labyrinth and tubule but the coelomosac did not show a trace of it. There seemed to be some accumulation in the hepatopancreas as the color was more intense in this organ (on adding a drop of acetic acid) than in the blood of equal depth. As the urine issues from the nephropore it is a light yellow and turns pink on the addition of acid. This shows that its pH is greater than 7.4.

It has already been noted that the phenol red experiments indicate a pH of about 7.5.

*"Indigo Carmine."*—Indigo carmine is composed of carmine blue and indigo disulfonate. The sample used had been passed by The Commission on Standardization of Biological Stains. It is long-known that indigo disulfonate is outwardly secreted by the vertebrate tubule. The dose was 0.7 cc. 80 mg. per cent per ca. 30 grams. Four hours after the injection the dye was markedly more concentrated in the bladder-contained urine than in blood even when collected only 25 minutes after the injection. On examination of the kidneys, no dye was found in the coelomosac or distal portion of the tubule. In one example concentrated dye was seen to leave the lumen of the proximal portion of the tubule upon application of pressure to the labyrinth, but there was no indication that the cells of the tubule take up the stain. The dye was concentrated in irregular patches in the labyrinth especially at the posterior end. There was no trace of it in the hepatopancreas and other tissues.

*Congo Red.*—Six-tenths of a cc. of 160 mg. per cent Congo red was injected into a 31-gram animal. Blood taken forty minutes later was a very light pink. The kidney was examined four hours after the injection; the coelomosac was a deep pink but the dye was absent from the rest of the nephron, and from the hepatopancreas, muscles, and gut.

*"Basic Fuchsin"* (aniline red; diamond fuchsin R.F.N.; magenta; passed by the C.S.B.S.).—The dose was 0.75 cc. 80 mg. per cent 30 grams. The animal was opened four hours after the injection. The stain had penetrated the muscles, hepatopancreas, coelomosac, nephric tubule, and other tissues. As compared with the blood, it was concentrated only in the labyrinth where it was a very intense purple. Soon after the injection the animals lay on their side in semi-torpor but recovered completely and removed all traces of dye from the blood.

*Acid Fuchsin.*—Eight-tenths of a cc. of 80 mg. per cent dye was injected into a 40-gram animal. The kidneys were examined after about 4.5 hours, at which time the dye was more concentrated in the bladders than in blood taken even 35 minutes after the injection. The labyrinth was a more intense pink than the blood seen through the same depth. The nephric tubule, coelomosac, hepatopancreas, muscles, and gut were not stained.

*Methylene Blue (Passed by the C.S.B.S.).*—Five-tenths of a cc. 50 mg. per cent were injected into a 22-gram animal. A blood-sample, taken after about two hours, was a very light blue. The animal was opened after about five hours. Methylene blue was concentrated only in the proximal portion of the tubule, where it existed as intracellular granules. After fixing the fresh kidney in 20 per cent formalin, the blue

concretions disappeared and the proximal portion of the tubule became a uniform blue. Evidently the intracellular granular condition depends upon an active process. The hepatopancreas and gills, but not the muscles, were merely stained. The experiment was repeated with identical results.

*Colloidal Carbon.*—Five-tenths of a cc. of "Higgins American India Ink: waterproof, black," diluted  $6 \times$  with crayfish-saline, was injected into a 20-gram animal. This was sufficient to give a very dark brown color to the blood. The animal was opened after four hours and the organs rinsed in situ with saline. The colloid had not penetrated any tissue. This is a functional demonstration of the absence of a nephrostome.

#### DISCUSSION

The primary question is whether filtration occurs through the nephron of the crayfish. The paper of Bethe, von Holst, and Huf (1935), which appears to furnish positive evidence for filtration, should be read with care, especially as certain investigators have taken their results at face-value. Bethe et al. augmented the internal hydrostatic pressure of the crab, *Carcinus maenas*, by a vertical column of saline which communicated with the haemocoel. The aqueous column then sank in abrupt steps, indicating a fall in the internal pressure. They stated that this is evidently a physiological event because raising the hydrostatic pressure after death resulted in only a slight fall of the column which they attributed to an expansion of the soft membranes of the integument. They also pointed out that if the crab dies during the experiment the column of saline either does not fall or sinks very slowly. The animals, which were observed in air, were stated to have shown a loss of fluid from three sites: (1) the gill-chambers; (2) the mouth; and (3) the nephropores. These investigators noted that the fluid from the gill-chambers contained protein but was cell-free; the writer thinks that this fluid may have issued partly from the mucus-secreting glands. Above all, the authors explicitly remarked that, during the fall of the aqueous column, generally no loss of liquid by way of the nephropores could be observed. Their suggestion that the kidneys of the crab regulate the internal hydrostatic pressure per se, i.e. even when the osmotic pressure does not vary, is therefore unfounded. As noted above, augmentation of the blood-volume by about one-third apparently does not increase the rate of urinary flow.

By measuring the oncotic pressure of the blood and the haemocoelic pressure of crayfish, Picken (1936) indicated that filtration is apparently possible.



The writer is not aware of any facts which can be taken as positive evidence for the filtration-resorption theory or against the absence of filtration and the outward secretion of liquid. Analogy with the vertebrate nephron is inadequate. Furthermore, outward secretion of liquid is known to occur in aglomerular fish (Marshall, 1930; Bieter, 1931). The urine of the latter is, like that of the crayfish, hypotonic to the blood. It is unknown, however, whether the hypotonicity of the urine of aglomerular forms is due to an outward secretion of a hypotonic liquid or to the elimination of an iso- or even hypertonic liquid, in the proximal part of the nephron, followed by a resorption of salts. Owing to phylogenetical reasons (see Marshall, 1934), the latter method does not appear probable.

There are several facts which indicate that the nephron of the crayfish is primarily if not entirely an organ of outward secretion:

1. There is no tenuous syncytium such as the glomerular capsule of the vertebrate nephron (Maluf, 1939, 1941a).

2. Large calcareous concretions sometimes occur in the lumen of the coelomosac, the most proximal organelle of the nephron, thus indicating that the coelomosac can secrete calcium (Maluf, 1941a). The coelomosac is also capable of accumulating Congo red (see above).

3. Experimental cytological evidence indicates an outward secretion of water by the distal half of the tubule (Maluf, 1941b).

4. Histologically there is no doubt that the labyrinth secretes material outwardly (Maluf, 1939). The labyrinthic cells are capable of accumulating and outwardly secreting cyanol, phenol red, indigo carmine, basic fuchsin, and acid fuchsin (see above).

5. The cells of the proximal portion of the tubule can accumulate methylene blue (see above). All parts of the nephron are therefore capable of secreting or accumulating one dye or another.

6. Inulin is outwardly secreted (see above).

7. From a teleological viewpoint the coelomosac is evidently not a filtration-organelle (Maluf, 1941a).

8. Injecting into a moderate-sized crayfish 1 cc. of crayfish-saline, i.e. a volume about one-third that of the initial blood-volume, and thus very probably increasing the internal hydrostatic pressure, does not augment the rate of urinary flow (Table I).

The Malpighian tubule of insects, as a result of physiological and cytological evidence, probably should be considered as an entirely secretory nephron. The beautiful live preparations of Wigglesworth (1931a, b, c) show that the Malpighian tubule can excrete fluid, in an apparently normal way, even under conditions when the hydrostatic pressure is zero.



The ingenious experiments of Patton and Craig (1939) show that the Malpighian tubule can absorb various isotonic salines isosmotically even when the hydrostatic pressure must be zero (the saline rose into the capillary gauge up to 10 to 15 mm. admittedly by capillarity). They also state that hydrostatic pressure does not cause an increase in "filtration" rate. It is not apparent to the writer why Patton and Craig assumed that the isosmotic uptake of solution by the Malpighian tubules is due to filtration. It is known that the alimentary epithelium of vertebrates absorbs solutions isosmotically and, at the same time, absorbs, selectively, ions of a particular species.

Kowalevsky (1889), Cuénot (1895), and Bruntz (1904) studied the affinity of the crustacean nephron, in situ, for ammonium carminate, indigo carmine, and certain other dyes. They did not indicate, however, whether the dyes were concentrated by the nephron because they made no statements as to the relative intensity of dye in the blood and urine. Kowalevsky and Bruntz noted that ammonium carminate and litmus stain the coelomosac but not the rest of the nephron while indigo carmine stains the tubule and labyrinth. Because the coelomosac stained red with litmus, Kowalevsky concluded that this organelle has an acid reaction. He also observed that the coelomosac, and not the labyrinth, has an affinity for Congo red and methylene blue. Cuénot believed that the labyrinth of the crayfish, lobster, and crabs has a *strongly alkaline* reaction (*italics his*) because it "energetically decolorised acid fuchsin"; the color reappeared on macerating the kidney in acetic acid. He also noted that alizarin violet (an alkaline dye) retains its color instead of going into the orange-red phase. I have found, on the other hand, that the labyrinth is capable of concentrating acid fuchsin and that treating the nephron with acetic acid does not augment the intensity of color. The dye was more concentrated in the urine than in the blood.

The present observations with the pH indicators, phenol red and neutral red, show that the cytoplasmic pH of the labyrinthic cells is about 7.0 and that the pH of the bladder-contained urine is about 7.5. Because the labyrinth will take up an acid dye, such as indigo carmine, is no reason to believe that its cells are basic. The uptake of dyes during life is not equivalent to the affinity of fixed dead tissues for dyes. This is a distinction which Kowalevsky and Cuénot did not make.

The statements of Kowalevsky and Cuénot that the labyrinth is alkaline led the writer (1938) to suggest that the nitrogenous products of protein-catabolism are outwardly secreted by the labyrinth. The facts that the labyrinth is not alkaline and that the concentration of the N-P-N is markedly lower in the urine than in the blood (see Delaunay, 1927 and 1931, for the crabs *Maia squinado* and *Cancer pagurus*; the crayfish has

not been studied with this regard) have greatly weakened that supposition. It should be borne in mind that *M. squinado* and *C. pagurus* are marine crabs without nephric tubules and eliminate a urine isotonic with their blood. It is therefore unlikely that the N-P-N is subjected to dilution by an outward secretion of water.

Because the main nitrogenous excretory product of the Crustacea is a highly diffusible substance,—ammonia (Delaunay, 1927, 1931), it seems probable that this escapes largely through the gills. Although we possess data on the over-all rate of ammonia-output by the crayfish (*Potamobius astacus*; see Brunow, 1911), there is no statement in the literature concerning the concentration of ammonia in the urine; consequently the rate of output of ammonia by the renal route is unknown. Partly because the concentration of ammonia is practically the same in the urine as in the blood of the above-mentioned crabs, it is possible that the existence of ammonia in the urine is merely due to diffusion.

#### SUMMARY

1. The techniques of measuring the rate of urinary flow and of collecting urine are described. The collection of urine from the nephropores by suction is a satisfactory procedure provided a correction is applied for the water lost by evaporation.

2. The techniques of collecting blood and of measuring renal clearances in the crayfish are described.

3. Raising the internal volume by one-third and therefore, presumably, augmenting the internal hydrostatic pressure, by the injection of 1 cc. of crayfish-saline, does not increase the rate of urinary flow.

4. Inulin and xylose will appear in the urine after being injected into the haemocoel. Glucose will occur in the urine provided enough is injected to permit its existence in the blood for a sufficient period.

5. The inulin-clearance and the  $U/P$  ratio of inulin vary inversely with the concentration of inulin in the blood. This demonstrates that inulin is secreted.

6. Inulin is not hydrolyzed by the hepatopancreas, kidneys, somatic muscles, or blood.

7. At low plasma-levels, the  $U/P$  ratios of xylose are very much below unity but rise above unity at high plasma-levels. This shows that xylose is either actively resorbed from a filtrate or is outwardly secreted but, with the low plasma-levels, at a relatively low rate compared with the secretion of water. The xylose-clearance:plasma-xylose curve is practically identical in shape with the  $U/P$ :plasma-xylose curve.

8. Although the renal clearances of xylose are much lower than the renal clearances of inulin, the plasma-concentration of the monosaccha-

ride falls more rapidly than that of the polysaccharide. This may be partly because the tissues can destroy xylose.

9. Only the labyrinthic cells can accumulate and outwardly secrete cyanol, phenol red, indigo carmine, basic fuchsin, and acid fuchsin. The coelomosac, but not the labyrinth or tubule, can accumulate Congo red. These dyes cannot accumulate in, and apparently do not penetrate into, other tissues of the body.

10. Only the cells of the proximal half of the tubule accumulate methylene blue.

11. Colloidal carbon does not enter the kidney; this is functional proof of the absence of a nephrostome.

12. The cytoplasmic pH of the labyrinthic cells is about 7; the pH of the bladder-contained urine is about 7.5.

13. The available facts (histological, chemical, physiological, and phylogenetical) indicate that the nephron of the crayfish is primarily if not entirely an organ of outward secretion.

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